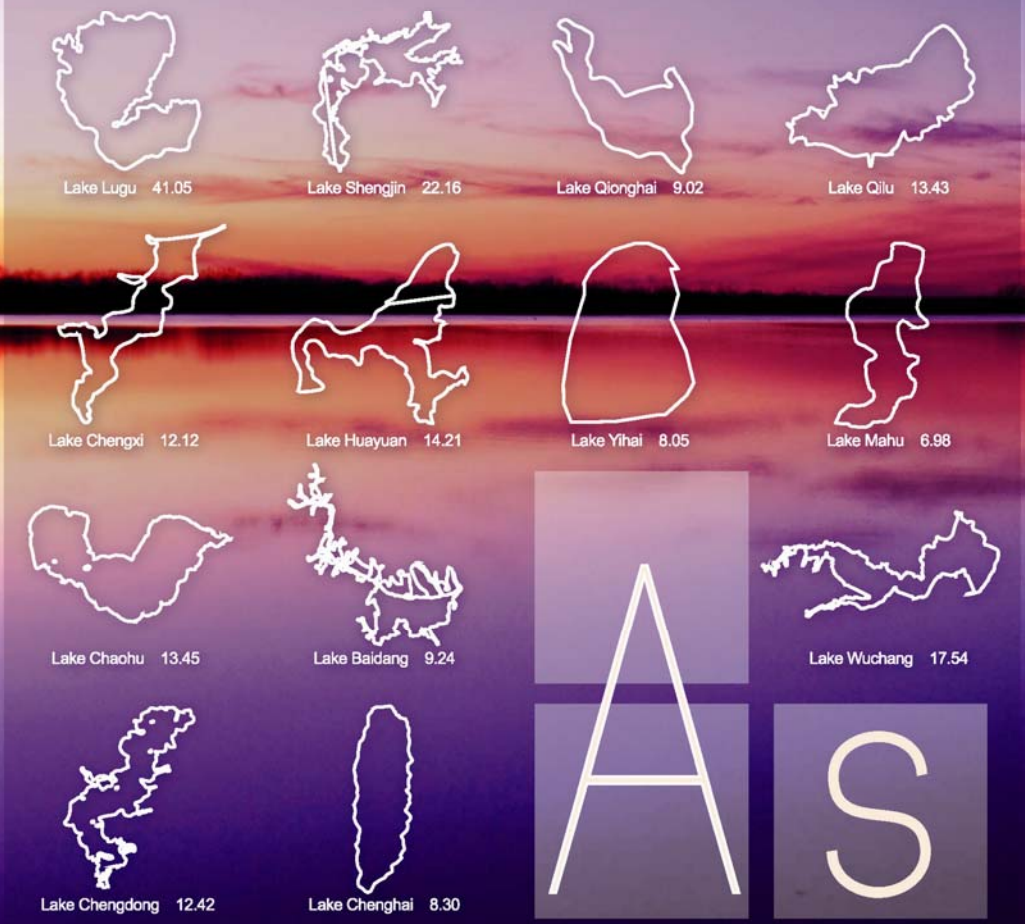
JES
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
October 1, 2014 Volume 26 Number 10
www.jesc.ac.cn



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Journal of Environmental Sciences Volume 26 Number 10 2014

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Experimental study using the dilution incubation method to assess water biostability

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ARTICLE INFO

Article history:
Received 4 November 2013
Revised 24 February 2014
Accepted 7 March 2014
Available online 4 August 2014

Keywords:
Water biostability
Heterotrophic bacteria
Bacterial growth rate
Dilution incubation method
Total growth organic carbon

ABSTRACT

Water biostability is of particular concern to water supply as a major limiting factor for heterotrophic bacterial growth in water distribution systems. This study focused on bacterial growth dynamics in the series dilution of water samples with TOC (total organic carbon) values determined beforehand. The results showed that the specific growth rate of *Pseudomonas fluorescens* P17 varied dramatically and irregularly with TOC value when TOC concentrations were low enough during the initial periods of incubation under given conditions. According to this relationship between bacterial growth rate and TOC, a dilution incubation method was designed for the study of water biostability. With the method under a given condition, a turning-point TOC value was found at a relatively fixed point in the curve between bacterial growth rate and TOC of water sample, and the variation of growth rate had different characteristics below the turning-point TOC value relative to that over this value. A turning-point TOC value similarly existed in all experiments not only with tap water, but also with acetate and mixed solutions. And in the dilution incubation method study, the affections were analyzed by condition factors such as inoculum amount, incubation time and nature of the organic carbon source. In very low organic carbon water environments, the variation characteristics of bacterial growth rate will be useful to further understand the meaning of water biostability.

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Introduction

Biostability is defined as the inability of water or material in contact with water to support microbial growth in the absence of a disinfectant (Rittmann and Snoeyink, 1984). Water biostability is of particular concern to water utilities as the major limiting factor for heterotrophic bacterial growth in water distribution systems. To achieve biological stability of drinking water in water distribution systems, two strategies have usually been accepted, which were maintaining enough residual disinfectant and producing high quality drinking water, and both of them require to assess water biostability first. Many parameters have been proposed to assess biostability of water, but the reliability and operational convenience still need improvement.

In the last decades, many determination methods for water biostability have been developed, which mainly measure the increase of bacterial biomass (AOC, assimilable organic carbon; MAP, microbially available phosphorus; BRP, bacterial regrowth potential) or the decrease of biodegradable dissolved organic carbon (BDOC) (Van der Kooij, 1982; Lehtola et al., 1999; Sathasivan and Ohgaki, 1999; Frías et al., 1995; Escobar et al., 2001).

Van der Kooij (1982) first proposed the concept of AOC, which was described as the organic compounds limiting growth of heterotrophic microorganisms. AOC bioassay is

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http://dx.doi.org/10.1016/j.jes.2014.07.011
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based on the linear relationship between AOC concentration and the bacterial batch maximum growth in water from inoculation to stationary phase. Generally, AOC value can be calculated by converting the bacterial maximum growth of water samples into acetate carbon (acetate-C) concentration according to a standard curve derived from the relationship between bacterial batch maximum growth and organic carbon concentrations of sodium acetate standard solutions. Lehtola et al. (1999) also put forward another biostability parameter, MAP, for phosphorus-limited water. MAP measurement was based on the linear relationship between the MAP value as the equivalent phosphate phosphorus (phosphate-P) and the bacterial batch maximum growth. Sathasivan and Ohgaki (1999) used an indigenous inoculum and took the amount of the maximum microorganism cells as the BRP value directly. AOC, MAP and BRP are all based on the relationship between substrate concentration and bacterial batch maximum growth from inoculation to the stationary phase, therefore, such bioassays are usually performed as "end-point" measurements, like the AOC bioassay (Hammes and Egli, 2005), in which a relative long-time bacterial batch culture process is necessary to get the maximum growth of bacteria. BDOC can be considered the portion of the DOC (dissolved organic carbon) that could be mineralized by heterotrophic microorganisms, given by the decrease in DOC in an inoculated sample after a given period of incubation (Frías et al., 1992; Joret and Levi, 1986; Lucena et al., 1990; Ribas et al., 1991; Servais et al., 1987, 1989). A number of methods have recently been developed to measure BDOC of water samples. The problem with most of these is the length of time (from days to weeks) required for the start up (colonization) and/or determination (Khan et al., 2003). AOC and BDOC are conceptually different; while the BDOC value corresponds to the difference between the initial DOC and the minimum DOC reached in the bacterial growth period, the AOC is the portion of DOC that can be converted to biomass (Huck, 1990), and so the parameters such as AOC are more often used to assess water biostability than BDOC.

Availability of carbon/energy sources and temperature are the two environmental factors that severely restrict heterotrophic growth in most ecosystems. Egli (2010) demonstrated that microbes had adapted and developed strategies to cope with the conditions at very low substrate concentration. One of these strategies is to perform a "multivorous" way of life by taking up and metabolizing dozens of different carbon substrates simultaneously; this "mixed substrate growth" equips the cell with a kinetic advantage and metabolic flexibility. These studies implied that the conditions at very low substrate concentration can stimulate the bacterial cell's assimilability of carbon sources, which makes the bacterial cell assimilate more kinds of carbon sources than those at normal substrate concentration. According to this concept, a bacterial cell can assimilate more kinds of carbon sources at very low substrate concentration than that at higher substrate concentration. Therefore, a method designed using a dilution strategy to very low substrate concentration, as in the present study, can reflect the bacterial assimilability to more kinds of carbon resources in water sample.

Yu (2006) developed an equation for microbial growth according to the thermodynamics of the microbial growth process as the following:

$$\Delta G = \Delta G^{O} - nRT \ln S + RT \ln \frac{\mu_{obs}}{\mu_{max} - \mu_{obs}} \quad (1)$$

where, ΔG is the total change of free energy of microbial growth, ΔG^0 is the standard free energy change, S is the molar concentration of substrate, n is a positive coefficient, μ_{obs} is the observed specific growth rate, μ_{max} is the maximum specific growth rate, R is gas constant in the Gibbs free energy function, and T is the temperature.

The function was based on the collision frequency theory for microbial growth (Button, 1998) and the assumption that cells had only a limited number of sites for taking up substrate (Alberts et al., 2001; Button, 1998; Hammes, 2000). In this article, the driving force of microbial growth, is influenced jointly by the molar concentration of substrate and the number of reactive sites on cells which can be described by the difference between the maximum specific growth rate (μ_{max}) and the observed specific growth rate (μ_{obs}) under given conditions as shown by the function above. This function indicates that the growth rate is not steady with variation of μ_{obs}, and depends on the conjunct effects of the molar concentration of substrate and the difference between μ_{obs} and μ_{max}, moreover, the effect of the difference between μ_{obs} and μ_{max} may be weakened by the increase of the substrate molar concentration, thus the growth rate will change more dramatically when the substrate concentration is very low, which was confirmed in our experimental study.

Eichinger et al. (2010) presented that increased bacterial growth efficiency with environmental variability emphasized the importance of cell maintenance in bacterial growth dynamics. It was found that the initial bacterial growth rate varied dramatically, and they contributed it to population synchronization as bacteria with cell maintenance were constrained by the presence or absence of food. By estimating specific activities of bacteria faced with a pulsed substrate supply with a 48-hr interval period, steady-state growth would be difficult to observe. O_2 concentration measurements revealed that respiration rates sharply increased as soon as substrate was introduced to the culture of low substrate concentration. This increase was so rapid that the potential O_2 consumption, due to the time lag among substrate addition in the culture, sampling, and respiration rate measurements, may be underestimated. This conclusion was certainly true for DOC measurements, because if bacterial respiration rates increased so rapidly, they obviously consumed DOC very quickly after substrate addition. Thus the low repeatability of initial growth and the fact that the growth changed more dramatically for fast DOC consumption under low substrate concentration can be well explained, which was also confirmed by our study, but the characteristics of the relationship between μ and TOC could be repeated under the same given conditions. The fast DOC consumption after substrate addition is necessary for bacteria to cope with the *in situ* heterogeneity of a largely oligotrophic and ever-changing environment, and may result from the uncoupling of anabolic and catabolic processes (del Giorgio and Cole, 1998).

The level of water biostability depends on a complex affection of factors, mainly including concentration of biodegradable organic carbon, water temperature, and residence time (Van der Kooij, 2000; Eichler et al., 2006; Lautenschlager et al., 2010). In the past decades, water biostability assessment has usually been based on the result of a long microbe growth period and TOC could not be used to draw conclusions on the organic compounds' growth-promoting potential, because the biological assimilability of a mixture of organic compounds depends on

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both the nature of the substances and the amount of carbon. In the current study, a dilution incubation method was designed to study water biostability (Egli, 2010; Yu, 2006; Eichinger et al., 2010). This work conducted a series of experiments to investigate the relationship between bacterial re-growth potential and TOC concentration under different conditions. The findings could be helpful for further understanding water biostability, designing determination methods and building criteria to define biostability.

1. Materials and methods

1.1. Bacterial strain and water samples

The inoculum bacterium was *Pseudomonas fluorescens* (P17), and its storage, revival, adaptation, and working stock culture were prepared according to the method of LeChevallier et al. (1993) with minor modifications. Briefly, the storage condition was a solution of 20% glycerol–2% peptone at −20 °C. Prior to use, the cultures were retrieved on R_2A agar, and incubated at 22 °C for 5 days. For adaptation, an isolated colony was inoculated into 100 mL of sterile, chlorine-neutralized tap water, which was then incubated at 22 °C for 7 days. An aliquot (0.1 mL) of the tap water-adapted culture was used to inoculate 100 mL of a sodium acetate solution with 2000 μg acetate-C/L. The sodium acetate solution was incubated at room temperature for 2–6 months. Bacterial counts of inoculum were generally in the range of 6.4×10^6 to 8.3×10^6 CFU/mL. The sodium acetate solution was used as a working stock culture to inoculate the dilution series of the water samples and the sodium acetate solution.

The study was carried out with three kinds of the water samples, which were the sodium acetate solution as standard substrate, stagnant tap water samples, and fish peptone solutions.

1.2. Preparation of TOC-free material

For the TOC determination and the dilution incubation method designed in the study, borosilicate glass vials (45 mL) with screw caps containing TFE-lined silicone septa were used. Carbon-free bottles and vials were rinsed with tap water, submerged in a 10% (wt/vol) potassium persulfate solution for 30 min, and rinsed with nanopure water 3 times before being heated at 60 or 260 °C in a constant temperature drying box for at least 8 hr. After rinsing the screw caps with nanopure water, they were soaked in a 10% (wt/vol) potassium persulfate solution at room temperature for 1 hr. Then, they were rinsed more than 3 times with nanopure water and finally air-dried at 60 °C.

1.3. Total adenosine tri-phosphate and TOC determination

To determine ATP, 100 μL water samples were mixed thoroughly with 100 μL of BacTiter-Glo™ solution (Promega Corporation, USA) and incubated at room temperature for 20 sec. Luminescence of the samples was measured in two kinds of luminometers (GloMax 20/20 and 96 GLO). The data were collected as absolute light units (AU) to calculate μ of P17.

TOC was measured as non-purgeable organic carbon with a total carbon analyzer TOC-5000(A) (Shimadzu, Japan). Aliquots of the water solutions (20 mL) filtered with a 0.22 μm filter were transferred into a C-free TOC vial. After stripping CO_2 with CO_2-free air, triplicate 50 μL samples were injected into the TOC analyzer for measurement. The TOC concentration of the sample was the average of the three measurements. To prepare a calibration curve a stock solution of sodium acetate solution with 2000 μg acetate-C/L was diluted by nanopure water to obtain solutions with carbon concentrations between 0 and 2000 μg acetate-C/L.

1.4. Dilution incubation method

After TOC determination, water samples filtered with a 0.22 μm filter were first diluted (most subjected to a series of successive two-fold dilutions) to a total volume of 10 mL in the borosilicate glass vials (45 mL) with the following buffer: 7.0 mg of K_2HPO_4, 3.0 mg of KH_2PO_4, 0.1 mg of $MgSO_4 \cdot 7H_2O$, 1.0 mg of $(NH_4)_2SO_4$, 0.1 mg of NaCl, and 1.0 μg of $FeSO_4$ (all

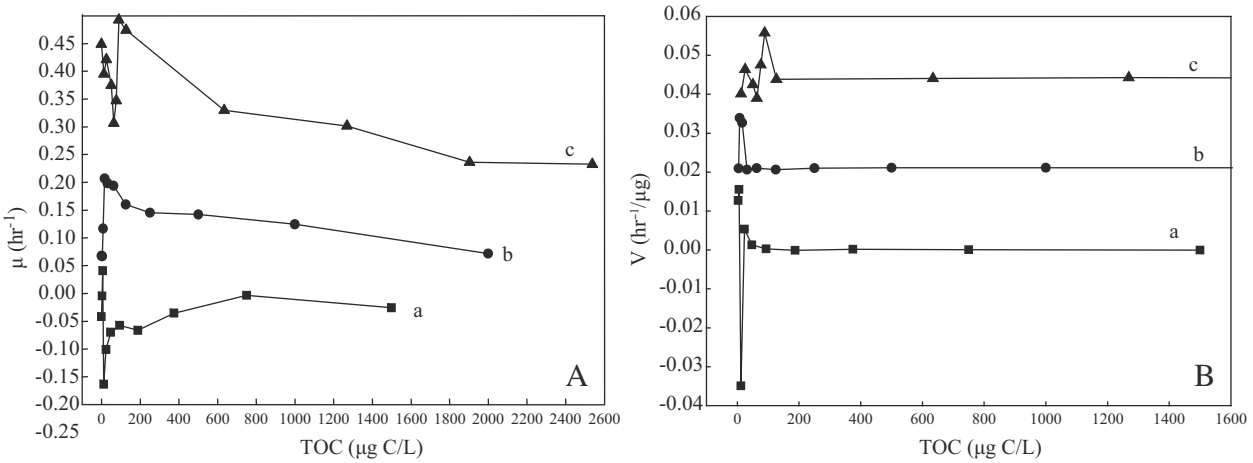

Fig. 1 – Relationships between specific growth rate (μ) and TOC (Total Organic Carbon) values (A), and V TOC values (B) (where, V is the slope between two adjacent TOC values in the curve between μ and TOC) of the acetate dilutions. (line a) 20 μL 6-month-culture inoculums with raw TOC concentration of 1500 μg C/L; (line b) 20 μL 6-month-culture inoculums with raw TOC concentration of 2000 μg C/L; (line c) 25 μL 3-month-culture inoculum with raw TOC concentration of 2538 μg C/L.

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quantities are per liter). In the series, the TOC value of the maximum dilution should be low enough to show fluctuating changes of the relationship between μ and TOC values of the diluted solutions. The diluted solutions were inoculated respectively with 20, 25, 50, 100, and 200 μL of the working stock culture, respectively, and then incubated at 30 °C for 5–30 min, 1 hr and 2 hr. Just before the specified time, the dilutions were shaken for 10 sec and then 0.1 mL was sampled for the ATP determination, and the two contiguous ATP values expressed in AU at the beginning (AU_0) and end (AU_1) of the incubation period were used to calculate the bacterial growth rate for the different incubation periods (Δt). The specific growth rate (μ) was calculated by Eq. (2):

$$\mu = \frac{\ln(AU_1/AU_0)}{\Delta t} \quad (2)$$

$$V = \frac{\mu_n - \mu_{n-1}}{TOC_n - TOC_{n-1}} \quad (3)$$

where, V is the slope between two adjacent TOC values in the curve between μ and TOC; n is the ordinal number of TOC determination points from the minimum to the maximum; TOC_n is the responding TOC value at the ordinal n point on X-axis of the curve; μ_n is specific growth rate value to TOC_n value; μ_{n-1} is specific growth rate before μ_n; TOC_{n-1} is TOC value before TOC_n.

2. Results and discussion

2.1. Relationship between μ and TOC values of the twofold dilution series of acetate solutions

Fig. 1 shows the results of the dilution incubation method with 20 and 25 μL inoculum per 10 mL acetate diluted solution for 1 hr incubation. Fig. 1A shows that the curve of the relationship between μ and TOC values of the acetate solution had a slope change when the TOC values decreased below the turning-point TOC value. The turning-point TOC value is the value at the point on the X-axis, which was the dividing point from the unsteady part to a relatively straight part on the curve of μ and TOC (or V and TOC). To make this change easy to find, Fig. 1A was converted into Fig. 1B by Eq. (3). The turning-points of TOC values were 11.72 (line a of Fig. 1B) and 15.625 μg C/L (line b of Fig. 1B) for 20 μL 6-month-culture inoculums with raw TOC concentrations 1500 and 2000 μg C/L, respectively, while it was 88.9 μg C/L (line c of Fig. 1B) with 25 μL 3-month-culture inoculum. The fluctuating changes of the growth were consistent with the growth change reflected by the thermodynamics of bacterial growth proposed by Yu (2006). The results showed that the smaller gradient of dilution should get a more accurate turning-point TOC value under given conditions. Although it was difficult to repeat the bacterial growth rate value at a certain substrate concentration under the given condition, the relationship characteristics could be repeated between μ and TOC or between V and TOC under a given condition with the dilution incubation method designed in the study, and these characteristics also were observed in mixed solutions such as the peptone solution or tap water (Fig. 2).

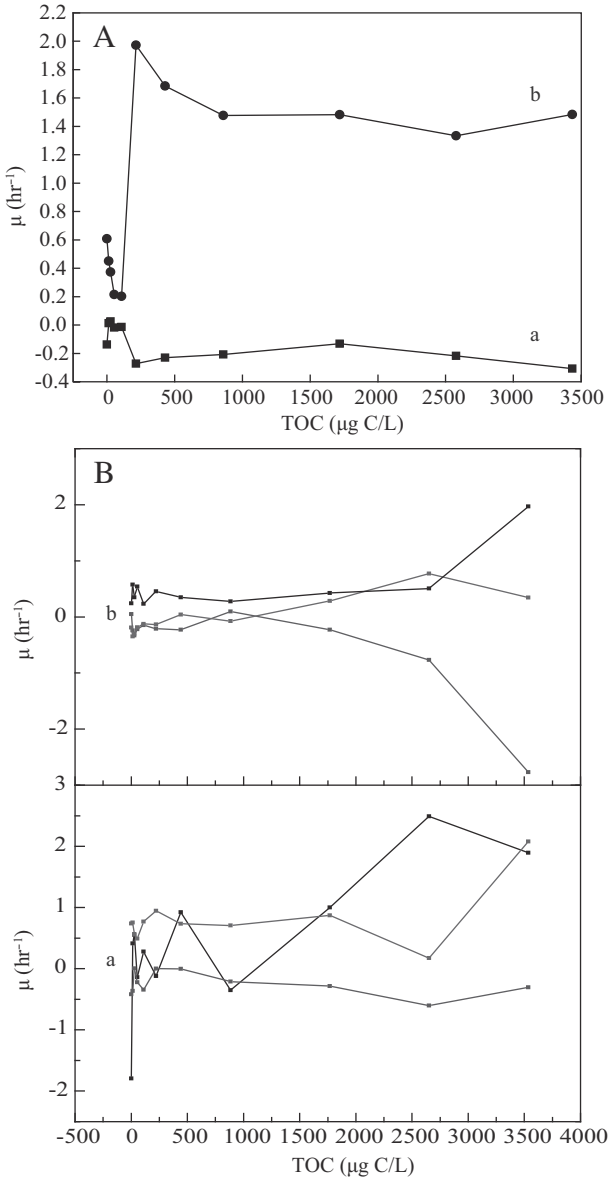

Fig. 2 – Comparison of the relationships between μ and TOC (Total Organic Carbon) values of the peptone dilutions (A) and the stagnant tap water dilutions (B) with 25 μL inoculum per 10 mL solution for 15 min (line a) and 1 hr (line b) incubation periods respectively.

2.2. Influences of incubation period and inoculation amount

Fig. 2A shows the turning-point TOC values of peptone solution, which were 214.8 or 1718 μg C/L for 15 min incubation period and 214.8 μg C/L for 1 hr incubation period. Fig. 2B shows the turning-point TOC values of stagnant tap water solution, which were 220.9 or 441.9 μg C/L for 15 min incubation period and were in the range of 220.9–883.7 μg C/L for 1 hr incubation period. Fig. 3 shows the turning-point TOC values of sodium acetate solution, which were 250, 15.625, and 62.5 μg C/L for 0.5, 1 and 2 hr incubation periods respectively. As regards the influence of incubation, the above results showed that the

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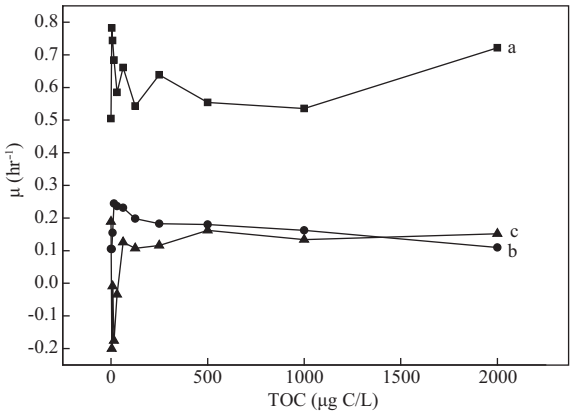

Fig. 3 – Comparison among the relationships between μ and TOC (Total Organic Carbon) values of the sodium acetate dilutions with 20 μL inoculum per 10 mL solution for the 0.5 hr (line a), 1 hr (line b) and 2 hr (line c) incubation periods respectively.

relationship between μ and TOC values for the 1 hr incubation period was more stable with a smaller turning-point TOC value in all incubation periods, therefore a 1 hr incubation period was the best choice for the bioassay, which might be due to the complex changes of bacterial growth and substrate concentration.

To further elucidate the relationship between the turning-point TOC values and water biostability, the amount of the inoculation culture was gradually increased from 20 to 200 μL (Fig. 4). The turning-point values shown with arrows on the curves were 31.25 μg C/L for 20 μL inoculum (Fig. 4A), almost 31.25 μg C/L for 50 μL inoculum (Fig. 4B) with more variations, 125 μg C/L for 100 μL inoculum (Fig. 4C) and 222.8 to 445.7 μg C/L for 200 μL inoculum (Fig. 4D). The results indicated that the turning-point TOC value increased with increasing inoculum amount. The result (Fig. 5) for peptone solution showed that the turning-point value (214.8 μg C/L) with 25 μL inoculum was smaller than that (234.7 to 939 μg C/L) with 50 μL inoculum. The explanation might be that more organisms to be supported require more organic carbon, and the turning-point TOC value might represent a limitation for bacterial growth. Hence, there might be some relationship between the turn-point TOC value and water biostability and it requires more study. The results in both Figs. 4 and 5 show that the larger amount of inoculum should not be chosen to obtain a more stable and accurate curve for the dilution incubation method. In this study, the best inoculum amount was 25 μL for 10 mL dilution solution.

2.3. *Comparison experiments with the standard sodium acetate solution*

Because the turning-point TOC value of water samples was found to be sensitive to the experimental conditions, a standard solution should be designed to obtain a basis for comparison. The AOC bioassay is designed with the sodium acetate solution as standard solution, so to convert the turning-point TOC value directly into an AOC value, the sodium acetate solution was accepted as the standard solution in the dilution incubation method. The first experiment was the comparison of peptone solutions and sodium acetate solution using the dilution

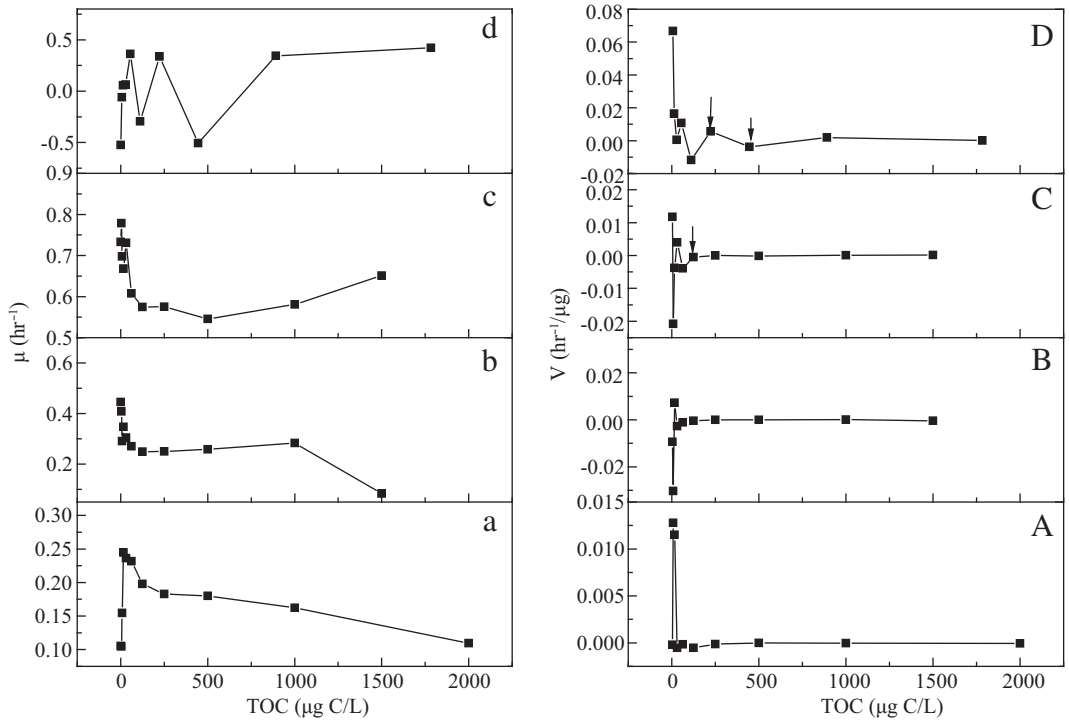

Fig. 4 – Comparison of the relationships between μ and TOC (Total Organic Carbon) values and the relationships between V (where, V is the slope between two adjacent TOC values in the curve and TOC values of the sodium acetate dilutions with 20 μL (a, A), 50 μL (b, B), 100 μL (c, C) and 200 μL (d, D) inocula respectively per 10 mL for 1 hr incubation period.

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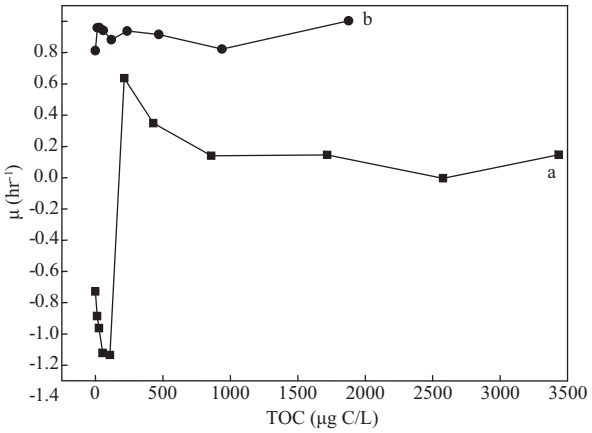

Fig. 5 – Comparison of the relationships between μ and TOC (Total Organic Carbon) values of the peptone dilutions with 25 μL (line a) and 50 μL (line b) inocula respectively per 10 mL for 1 hr incubation period.

incubation method under the same conditions. The second experiment was the comparison of the stagnant tap water sample and sodium acetate solution. The results are shown in Table 1. The experimental result of the comparison of the peptone solution and the sodium acetate solution as standard solution for the method, showing that the turning-point TOC value (214.8 μg C/L) of the peptone solution was clearly larger than that (88.9 μg C/L) of the standard solution. The turning-point TOC values (204.26 and 204 μg C/L) of the stagnant tap water solution were obviously larger than those (55.7 μg C/L) of the standard solution (acetate solution). In the second experiment, the turning-point TOC values of the stagnant tap water sample showed high repeatability. The TOC values of both the peptone solution and stagnant tap water solution were converted into equivalent values (1422.1 μg C/L for peptone solution; 445.6 μg C/L for stagnant tap water sample) as acetate-C (acetate carbon) by Eq. (4).

$$\text{Acetate-C} = \text{TOC} \times \frac{\text{TOC}_t}{\text{TOC}_{t0}} \quad (4)$$

where, TOC is total organic carbon of the water sample; TOC_t is the turning-point TOC value of the water sample; TOC_{t0} is the turning-point TOC value of the standard solution.

Table 1 – Results of the dilution incubation bioassay of the water samples.

Experiment	Water sample	TOC (μg C/L)	TOC_t value of water sample (μg C/L)	Acetate-C (μg C/L)
1	Peptone solution	3436	214.8	1422.1
	Acetate solution	2538	88.9	
2	Tap water	1632	204	445.6
	Tap water	1634	204.26	445.6
	Acetate solution	891.5	55.7	

TOC (Total Organic Carbon) is the TOC value of the raw water sample without dilution; TOC_t is the turning-point TOC value of the water sample.

Therefore in causing the same growth changes on the curve of μ and TOC, the acetate-C value of peptone was 1422.1 μg C/L, which was more than the 445.6 μg C/L value of the stagnant tap water sample, which should be related to the assimilability of the organic carbon. The turning-point TOC values (88.9, 55.7 μg C/L) of the standard solutions were equal to the same AOC values with higher biostability, which implied that a water sample with TOC value below the turning-point TOC value had higher biostability.

3. Conclusions

In this study, the dilution incubation method was designed to be applied in the research of water biostability, combining bioassay and chemical TOC analysis based on bacterial growth dynamics in water. The experimental studies on bacterial growth dynamics demonstrated that growth change occurred simultaneously when the starved bacteria contacted substrate. It was found that there was a special growth rate change below a turning-point TOC value on the relationship curve between μ and TOC of the water sample. The turning-point TOC value is the value at the point on the *X*-axis where the straight curve became a fluctuating curve of μ and TOC (or *V* and TOC). It was found that the turning-point TOC value was related to inoculum amount, bacterial age, and incubation period. The turning-point TOC value would increase when the amount of inoculum increased, and a 1 hr incubation period with 25 μL inoculum was selected in the method to get better results. The experimental results showed some changes of the turning-point TOC value with the kind of water samples such as tap water samples and peptone solution, and compared the results with that of the standard sodium acetate solution. By taking sodium acetate solution as the standard solution, a relationship was built between the turning-point TOC value of sodium acetate solution and the AOC value. The turning-point TOC values (88.9, 55.7 μg C/L) of the standard solutions were equal to the same AOC values with higher biostability, which implied that a water sample with TOC value below the turning-point TOC value had higher biostability. Therefore, the dilution incubation method has the potential to further studies on water biostability.

Acknowledgments

We are grateful to the National Natural Science Foundation of China for their financial support (No. 51378374), and the Fundamental Research Funds for the Central Universities (No. 0400219207).

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Journal of Environmental Sciences (Established in 1989)

Vol. 26 No. 10 2014

Supervised by	Chinese Academy of Sciences	**Published by**	Science Press, Beijing, China
Sponsored by	Research Center for Eco-Environmental		Elsevier Limited, The Netherlands
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ISSN 1001-0742